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The post-illumination chlorophyll fluorescence transient indicates the RuBP regeneration limitation of photosynthesis in low light in Arabidopsis

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ABSTRACT

The mechanism of post-illumination chlorophyll fluorescence transient (PIFT) was investigated in Arabidopsis. PIFT was detected in the wild type after illumination with low light. In the *fba3-2* (*fructose-1,6-bisphosphate aldolase*) mutant, in which PIFT is enhanced, strong light also induced PIFT. PIFT was suppressed not only in the triose phosphate/phosphate translocator (*tpt-2*) mutant, but also in *tpt-2 fba3-2*, suggesting that triose phosphates, such as dihydroxyacetone phosphate (DHAP), are involved in the PIFT mechanism. We concluded that PIFT is associated with ribulose-1,5-bisphosphate (RuBP)-regeneration limitation of photosynthesis in low light.

1. Introduction

In photosynthesis, electrons are transported in the thylakoid from photosystem (PS) II to PSI and finally to $NADP^+$ in the stroma. Electron transport results in the generation of the transthylakoid proton motive force that drives ATP synthesis. The products, NADPH and ATP, are utilized via the Calvin cycle to fix CO_2 into triose phosphate, which is then converted to carbohydrates such as starch and sucrose [1]. A major goal of research into photosynthesis has been to elucidate the mechanisms controlling the sequence of reactions.

Investigation of the slow kinetics of changes in chlorophyll *a* fluorescence, i.e., in the time range of seconds to minutes, is one of the most effective ways of analyzing the regulation of photosynthesis, and has been applied to a wide range of plants and algae. In contrast, although post-illumination chlorophyll fluorescence transient (PIFT) (Fig. 1) is equally characterized by slow kinetics of chlorophyll fluorescence, it has not attracted much attention, and the mechanism has not yet been fully elucidated. However, PIFT is highly reproducible and responds sensitively to environmental conditions such as actinic light intensity and temperature [2,3], suggesting that PIFT has a potential as a new tool for investigating how photosynthesis is regulated.

PIFT has occasionally been described in the literature: (i) it is proposed, based on in vitro data, to be induced by reverse reactions in the Calvin cycle from 3-phosphoglycerate (3-PGA) to DHAP [4,5]; and (ii) PIFT is absent in tobacco mutants lacking the chloroplastic NAD(P)H dehydrogenase (NDH) complex [6–9]. It is therefore possible to assume that PIFT is induced by accumulation of DHAP during a previous light period, which is re-metabolized through the reverse reactions in the Calvin cycle and subsequently results in the release of NADPH, which can donate electrons to plastoquinone (PQ) in the reaction mediated by the NDH complex. In the presence of reduced PQ, the yield of chlorophyll fluorescence, excited by weak modulated measuring light, rises due to disruption of the redox equilibration between Q_A and Q_B, the primary and secondary quinone acceptors in PSII, respectively.

We have recently reported that an Arabidopsis mutant, *fba3-2*, exhibits enlarged PIFT [10]. Fructose-1,6-bisphosphate aldolase (FBA) utilizes DHAP as a substrate in the Calvin cycle. Therefore, *fba3-2* makes it possible to examine the above scheme for PIFT in vivo. In this study, we focused on the involvement of DHAP in PIFT and attempted to analyze the regulation of photosynthesis on the basis of the results obtained. PIFT was suggested to be caused by DHAP accumulation behind the RuBP regeneration limitation in the Calvin cycle in low light.

2. Materials and methods

Arabidopsis thaliana wild type (Columbia *gl1* background), an EMS-derived mutant of *fba3-2* (Col-*gl1* background, previously

Abbreviations: FBA, fructose-1,6-bisphosphate aldolase; PIFT, post-illumination chlorophyll fluorescence transient; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; TPT, triose phosphate/phosphate translocator

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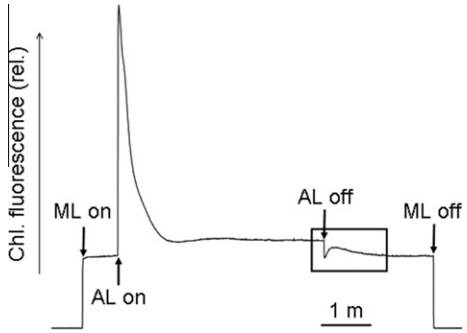

Fig. 1. Post-illumination chlorophyll fluorescence transient (PIFT). Chlorophyll fluorescence induction curve in a dark-adapted leaf of Arabidopsis wild type under illumination with actinic light (AL, 50 μmol photons $m^{-2}s^{-1}$) and subsequent PIFT (boxed area) are shown. The measurements were made using measuring light (ML) of approx. 0.1 μmol photons $m^{-2}s^{-1}$ under normal air conditions. The data are from Fig. 1b in [10].

termed *fba3-1*, [10]), and a T-DNA insertion mutant, *tpt-2*, (Col-0 background, SALK_073707) [11] were used. *tpt-2* contains a insertion downstream of the start ATG and an allele of the previously identified *tpt-1* mutant with insertions 24 bp upstream of the start ATG [12,13]. The plants were grown in soil under growth chamber conditions (50 μmol photons $m^{-2}s^{-1}$, 50% RH, 16 h-light/8 h-dark cycle, 23 °C). A homozygous T-DNA insertion was verified by PCR analysis using the T-DNA-specific primer (Lba1) 5′-GGTTCACG-TAGTGGGCCATCG-3′ and gene-specific primers for *TPT*: 5′-CCAAA TCGAGATCATCGTAGC-3′ (forward) and 5′-CTTTTCTCTCCTCCGTT-GATC-3′ (reverse). Total RNA extraction and RT were performed as described previously [10]. RT-PCR was performed using the *TPT*-specific primers 5′-TTCATCTCTGATCTCTTCTGG-3′ (forward) and 5′-CGTTTCTCTTCTTCGATCTTG-3′ (reverse). *ACTIN8* transcripts were analyzed using the primers 5′-GAGAGATTCAGGTGCCCAG-3′ (forward) and 5′-AGAGCGAGAGCGGGTTTTCA-3′ (reverse). Modulated chlorophyll fluorescence was measured using a mini-PAM fluorometer (H. Walz, Germany) as described previously [10]. The magnitude of PIFT was normalized as $(F_{peak} - F'_0)/F'_0$, where F_{peak} and F'_0 are the fluorescence peak level during the transient and the minimum level after actinic illumination, respectively. DHAP, sucrose, and starch contents were determined as described previously [14–16].

3. Results

3.1. Effects of light intensity on PIFT in the wild type and fba3-2 mutant

Fig. 1 shows a typical result of measurement of PIFT in a wild type leaf. PIFT can be detected for approx. 1 min after cessation of actinic illumination (Fig. 1, boxed area). PIFT was restricted to low light regions from approx. 24–120 μmol photons $m^{-2}s^{-1}$ as previous illumination, and was not detected after illumination of light with intensity higher than this or as low as 8 μmol photons $m^{-2}s^{-1}$ in the wild type (Fig. 2A). In *fba3-2*, PIFT was detected at all the light intensities used except at the lowest intensity (Fig. 2A).

3.2. PIFT in tpt-2

To investigate the involvement of DHAP in PIFT, we measured DHAP content in a leaf in low light: it was indeed higher in *fba3-2* than in the wild type (Table 1). However, it was impossible to reproduce PIFT in intact chloroplasts, ruling out any further attempts to examine the relationship between PIFT and DHAP accumulation. We therefore used *tpt-2* (Fig. 2A). As discussed later, to investigate PIFT, especially in *fba3-2*, it seemed important to focus

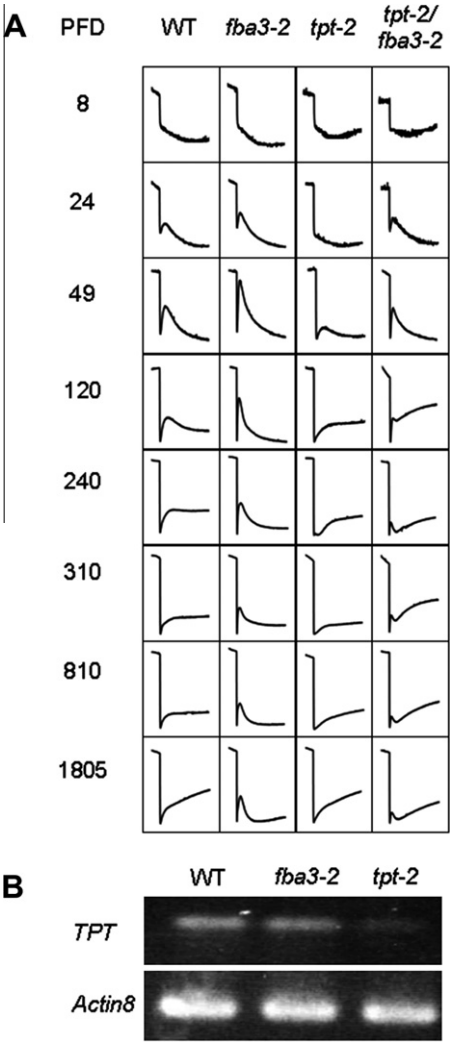

Fig. 2. Analysis of PIFT. (A) Dependence of PIFT on the intensity of previous illumination. The measurents were made as in Fig. 1. in the wild type (WT), *fba3-2*, *tpt-2*, and *tpt-2 fba3-2* using light at various intensities (PFD, μmol photons $m^{-2}s^{-1}$). (B) RT-PCR for *TPT* in the wild type (WT), *fba3-2*, and in *tpt-2*. *ACTIN8*, loading control.

Table 1
DHAP content in a leaf in low light of approx. 50 μmol photons $m^{-2}s^{-1}$. The data represent the mean ± S.D. of at least three experiments.

Plant	DHAP (nmol per g fresh weight)
Wild type	19.49 ± 1.57
fba3-2	23.35 ± 1.97
tpt-2	13.69 ± 1.19

on a factor residing outside the Calvin cycle, such as triose phosphate/phosphate translocator (TPT). It is of note that (i) leaf DHAP content in low light was lower in *tpt-2* than in the wild type (Table 1), and (ii) a mutant line impaired in the Calvin cycle enzyme triose phosphate isomerase (TPI) (SALK_026097, At2g21170), which catalyzes direct production of DHAP in the cycle, was considered to be a desired material here and indeed did not exhibit PIFT; however, the mutant could not grow photoautotrophically, making the results uncertain (data not shown).

Gene disruption in *tpt-2* was not complete, and residual expression was present (Fig. 2B). In *tpt-2*, PIFT was more significantly suppressed than in the wild type (Fig. 2A). To see more clearly the effect of the genetic background of the *tpt-2* mutant, a double mutant *tpt-2 fba3-2* was generated by crossing each single mutant and

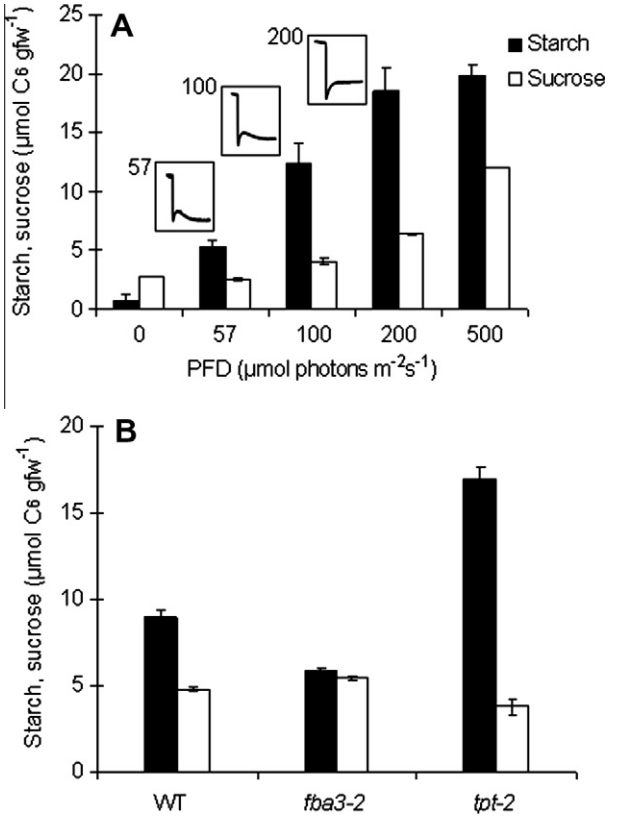

Fig. 3. Carbohydrate content in a leaf. (A) Effects of light intensity on starch (black bar) and sucrose (white bar) content in the wild type. Plants grown at approx. 50 μmol photons $m^{-2}s^{-1}$ were illuminated with light at various intensities for 3 h, after which leaf starch and sucrose content were measured. Inset: PIFT representative of the corresponding conditions. Measurements were made before the 3 h-treatment by light exposure. (B) Starch (black bar) and sucrose (white bar) content in the wild type (WT), *fba3-2* and *tpt-2*. Measurements were made after 3 h illumination at approx. 50 μmol photons $m^{-2}s^{-1}$. The data points represent the mean ± S.D. of at least three experiments.

screening the resulting F_2 generation for homozygous double mutants. The large PIFT, i.e., the trait in *fba3-2*, was suppressed significantly, as shown in *tpt-2 fba3-2* (Fig. 2A).

3.3. Effects of altered photosynthate partitioning on PIFT

Fig. 3A shows the relationship between appearance of PIFT and the carbohydrate content in a wild type leaf. Starch content increased with increasing light intensity and reached a plateau or saturation at 200 μmol photons $m^{-2}s^{-1}$. Sucrose content appeared to increase significantly at intensities above the saturation point for starch accumulation. At lower light intensities, sucrose content was similar to that observed in leaves placed in the dark. PIFT was detected in such lower light conditions. These results suggest that alternation in photosynthate partitioning affects PIFT.

To bridge the gap between photosynthate partitioning and the development of PIFT, we analyzed the carbohydrate content in *fba3-2* and *tpt-2*. Consistent with the distinct PIFT in these mutants (Fig. 2A), clear contrasts in both starch and sucrose content were observed: the starch content was lower in *fba3-2* than in the wild type, and higher in *tpt-2*, and the sucrose content was higher in *fba3-2* and lower in *tpt-2* (Fig. 3B).

4. Discussion

Infra-red light, which preferentially excites PSI, suppressed PIFT completely in the wild type and *fba3-2*, suggesting that PIFT is induced by PQ reduction [10]. As to the origin of reducing power for the PQ reduction, Mano et al. [5] reported that PIFT can be artificially enhanced by adding DHAP to a chloroplast suspension during PIFT: they interpreted the phenomenon as a reverse reaction in the Calvin cycle from DHAP, via glyceraldehyde 3-phosphate (GAP) and 1,3-bisphosphoglycerate (1,3-PGA), to 3-PGA catalyzed by TPI, GAP dehydrogenase, and phosphoglycerate kinase, during which reducing power NADPH is reproduced. We also assume DHAP to be a key substrate for PIFT, for the following reasons.

In *fba3-2*, PIFT was enlarged (Fig. 2A). Because FBA uses DHAP as a substrate, it is not surprising if DHAP accumulates excessively in *fba3-2*. Antisense potato plants with reduced plastid aldolase activity have been produced [17,18] in which DHAP levels indeed increased with decreasing FBA activity [17]. In the antisense plants, photosynthesis and growth were significantly inhibited due to the low rate of RuBP regeneration caused by suppressed FBA activity. Similarly, the total aldolase activity was reduced to approx. 80% of the wild type level in *fba3-2*, and the overall rate of photosynthetic electron transport declined to the same extent [10]. On the other hand, transitory starch accumulated less in *fba3-2* than in the wild type, while sucrose accumulated more in *fba3-2* than in the wild type (Fig. 3B). These results can be explained by the fact that FBA catalyzes the reaction at the branching point of the Calvin cycle, i.e., one leads to the RuBP regenerative phase that further branches off to the starch synthesis pathway and the other leads to triose phosphate export from the chloroplast for sucrose synthesis in the cytosol. The increased sucrose synthesis in *fba3-2* would require increased accumulation of DHAP, as suggested by the increased DHAP content in the leaf in *fba3-2* (Table 1). We conclude that the enhanced PIFT in *fba3-2* is attributable to the anticipated increase in DHAP.

PIFT was suppressed in *tpt-2*, and even the enlarged PIFT in *fba3-2* was also diminished in the *tpt-2* background (Fig. 2A). In *tpt-2* the starch content was higher than in the wild type and the sucrose content was lower (Fig. 3B). This is a typical response of Arabidopsis to diminished TPT activity [12,13]: increased starch synthesis is considered to compensate for decreased export of triose phosphate via TPT and thus decreased sucrose synthesis in the cytosol. Does this typical response cause a decline in DHAP content in the stroma? Antisense repression of TPT activity in potato has revealed that TPT deficiency results in a large decrease in phosphate in the stroma (due to inhibition of its import from the cytosol) and an increase in 3-PGA (probably due to the resultant short supply of ATP to the Calvin cycle) [19]. The resulting high ratio of 3-PGA/phosphate is known to activate the key enzyme for starch synthesis ADP-glucose pyrophosphorylase (AGPase) [20]. Activation of AGPase promoted starch synthesis on the one hand, but on the other hand, this unusual active starch synthesis was reported to exhaust the DHAP pool in the stroma [19]. We concluded that the suppressed PIFT in *tpt-2* and *tpt-2 fba3-2* is attributable to the anticipated decrease of DHAP, which is suggested by the decreased DHAP content in the leaf in *tpt-2* (Table 1).

We have attempted to seek the factors that affect the enhanced PIFT in *fba3-2* by crossing with relevant mutants defective in, e.g., PsbS protein involved in the development of non-photochemical chlorophyll fluorescence quenching (*npq4*, [21]) [10] and NADP-malate dehydrogenase protein mediating export of excess reducing equivalents from the chloroplast (SALK_012655, At5g58330) (data not shown). However, the enhanced PIFT was robust, and did not alter in any cases. The one exception was the mutant lacking the NDH complex (*ndhM*) [22]: in *ndhM fba3-2*, PIFT was almost completely abolished, probably due to inhibition of electron donation to the PQ pool via the NDH complex [10]. These observations suggest that PIFT is specific to the proposed mechanism, i.e., the electron reverse flow in the Calvin cycle that is connected to the PQ

pool via DHAP and the NDH complex. *tpt-2* and *fba3-2* provide us a rare opportunity to analyze this flow.

PIFT was observed in low light in the wild type. It is generally thought that the rate of photosynthesis is limited in low light by the rate of RuBP regeneration in the Calvin cycle, which is itself determined by the efficiency of light harvesting by the photosystems and/or the rate of electron transport in the thylakoid [1]. The RuBP regenerative phase in the Calvin cycle initiates from fructose-1,6-bisphosphate, which is produced by the reaction catalyzed by FBA using DHAP and GAP as substrates. The limitation of RuBP regeneration in low light may therefore mimic the slowed RuBP regeneration in *fba3-2*. We conclude that PIFT in low light in the wild type is induced by DHAP accumulation due to the inherent limitation of RuBP regeneration in the Calvin cycle. However, this DHAP accumulation in low light may not be significant as judged by the small PIFT in the wild type (Figs. 1 and 2A) and it may be suppressed or abolished in response to the switch from starch synthesis to sucrose synthesis with increasing irradiance (Fig. 3A). In *fba3-2*, the impaired FBA activity might, at all light levels, produce a situation similar to RuBP regeneration limitation in low light in the wild type (Fig. 2A).

It should be noted that after exposure to high light in low CO_2 there appears to be another PIFT component in *fba3-2* that occurs with distinct kinetics after the PIFT described in this study. Schreiber [4] has reported that addition of DHAP to a chloroplast suspension results in production of ATP via reverse reactions in the Calvin cycle and subsequent hydrolysis of ATP by the ATPase in the thylakoids, followed by reverse electron flux in the thylakoid that reduces PQ. This may be a complicating factor in PIFT kinetics.

There are two other options for PIFT: one is characterized by quenched F'_0) level termed F'_0) and the other is by F'_0) equal to F_0. Investigation of the three (or four) types of PIFT will provide insight into in vivo regulation of photosynthesis. In conclusion, we tentatively assume that PIFT as presented here is associated with pure RuBP regeneration limitation in low light.

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